ASBMT
American Society for Blood and Marrow Transplantation

Interferon Gamma 13-CA-Repeat Homozygous Genotype and a Low Proportion of CD4$^+$ Lymphocytes Are Independent Risk Factors for Cytomegalovirus Reactivation with a High Number of Copies in Hematopoietic Stem Cell Transplantation Recipients

Emilia Jaskula,[1] Dorota Dlubek,[1,2] Dorota Duda,[2] Katarzyna Bogunia-Kubik,[1] Anna Mlynarczewska,[1] Andrzej Lange[1,2]

Cytomegalovirus (CMV) reactivation was analyzed in 92 recipients of allogeneic hematopoietic stem cell transplantation (HSCT) in relation to the proportion of CD4$^+$ lymphocytes in blood and a microsatellite polymorphism within the first intron of the interferon-gamma (*IFNG*) gene. CMV reactivation was found in 50% of the HSCT recipients; in 30% of these individuals, the level of CMV copies exceeded 100 per 10^5 peripheral blood (PB) cells on at least one occasion during the 100-day post-HSCT observation period. This high CMV copy level was most frequently found between 31 and 60 days post-HSCT ($P = .021$). Patients with $\geq$ 100 CMV copies/10^5 cells were characterized by poorer overall survival (OS) compared with those lacking CMV copies or having $<$ 100 CMV copies/10^5 cells ($P = .04$), and they suffered from severe post-HSCT complications, including acute graft-versus-host disease (aGVHD) and relapse. Thus, patients with $\geq$ 100 CMV copies/10^5 cells were designated as having clinically significant CMV reactivation. Patients with $<$ 10% CD4$^+$ lymphocytes had a higher number of CMV DNA copies than those with higher proportions of CD4$^+$ lymphocytes (0.62 vs 0.21, $P = .001$; mean $\pm$ SEM, 4422 $\pm$ 1667 vs 937 $\pm$ 662 CMV copies/10^5 cells, $P < .001$, for the proportion of cases with reactivation and numbers of copies, respectively). Similarly, patients carrying 2 *IFNG* 13-CA-repeat alleles (homozygotes) had more frequent CMV reactivation (0.50 vs 0.26; $P = .039$) and a higher CMV load (4111 $\pm$ 1699 vs 950$\pm$591 CMV copies/10^5 cells; $P = .041$) compared with those with other *IFNG* microsatellite allele constellations. Multivariate analysis demonstrated that the *IFNG* 13-CA-repeat homozygous genotype (odds ratio [OR] = 0.221; $P = .044$), a low proportion of CD4$^+$ lymphocytes (OR = 0.276; $P = .050$), and a lack of optimal (10/10 alleles) donor–recipient HLA match (OR = 15.19; $P = .006$) were independent risk factors for CMV reactivation with a high number of copies.

Biol Blood Marrow Transplant 15: 1296-1305 (2009)

KEY WORDS: Cytomegalovirus, Interferon-gamma genotype, CD4$^+$ lymphocyte

INTRODUCTION

Cytomegalovirus (CMV) belongs to the *Betaherpesvirinae* subfamily of *Herpesviridae*. Between 50% and 85% of healthy adult individuals have IgG anti-CMV antibodies, which result from previous exposure to CMV [1]. Following the initial exposure, CMV resides in the host cells and is controlled by the T cell immune response. CMV reactivation occurs most frequently in immunosuppressed individuals, including those receiving hematopoietic stem cells (HSCs). CMV reactivation is associated with 30% of cases of CMV infection; these cases usually respond poorly to treatment and carry a high mortality rate [2].

Interferon-gamma (IFNG) is an antiviral factor that exerts an inflammatory response against CMV-infected cells [3]. IFNG generation potential is correlated with the number and proportion of CD4+ lymphocytes [4]. Moreover, IFNG production is known to be influenced by some HLA-associated features and also by *IFNG* gene polymorphism [5,6]. Two

From the [1]Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wroclaw, Poland; and [2]Lower Silesian Center for Cellular Transplantation with National Bone Marrow Donor Registry, Wroclaw, Poland.

Financial disclosure: See Acknowledgments on page 1303.

Correspondence and reprint requests: Andrzej Lange MD, Institute of Immunology and Experimental Therapy, Polish Academy of Science, Department of Clinical Immunology, Rudolf Weigl 12, 53-114 Wroclaw, Poland (e-mail: lange@iitd.pan.wroc.pl).

Received February 18, 2009; accepted June 10, 2009

1083-8791/09/1510-0010$36.00/0
doi:10.1016/j.bbmt.2009.06.008

polymorphic features—a single-nucleotide polymorphism (A/T) within the first intron of the *IFNG* gene at position +874 from the translation start site and a microsatellite polymorphism of CA repeats (11-15) starting at position +875—are closely linked to one another. Good IFNG producers are considered to be individuals with 12 CA repeats and a T at the polymorphic site [7,8]. Other microsatellite alleles are associated with the presence of A, but it seems that only those with 13 repeats have rather low IFNG generation potential [9,10]. The literature contains conflicting reports with respect to this observation, however [11]; in other words, whether a microsatellite polymorphism or the presence of an A/T transition can influence the gene transcription, thus making an individual more or less adept at IFNG production, remains up for debate. In the present study, we identified patients who were more susceptible to CMV reactivation than those receiving transplants from donors not optimally matched at the allelic level and having a low CD4+ lymphocyte proportion and count. We found a significant association between recipient low IFNG generation microsatellite genotype (13-CA-repeats) and the risk of CMV reactivation at a clinically relevant level.

PATIENTS AND METHODS

Patient Characteristics

Over a 4-year period (2004-2008), 92 patients underwent HSC transplantaion (HSCT) (45 with a transplant from a sibling donor and 47 with a transplant from an alternative donor). Conditioning regimens that included antithymocyte globulin (ATG) or Campath (Table 1) were applied to 70 patients who also received gancyclovir during the conditioning regimen to counter the increased risk of viral reactivation [12]. All patients with ≥ 100 CMV copy numbers/10^5 peripheral blood (PB) cells (except 3 with a smooth post-HSCT course) were given a 14-day course of gancyclovir.

All patients were routinely followed up with respect to clinical complications and blood tests, including the proportions of $CD4^+$ lymphocytes in blood and the number of CMV DNA copies. The latter analyses were performed at 1-week intervals until 30 days post-HSCT, then monthly until 100 days post-HSCT, as well as when clinical symptoms were suggestive of CMV reactivation (ie, fever of unknown origin associated with marrow depression and/or elevated aminotransferases). The number of measurements obtained varied from 3 to 27 (median, 8). Patients with higher numbers of measurements were usually positive for CMV copies and were further followed to monitor the effect of the preemptive treatment. Thus, a lower number of measurements reflects a smooth post-HSCT course. Fourteen patients died before 100 days post-HSCT; of these, 9 were positive for herpes virus reactivation.

Table 1. Patient Characteristics

Number of patients	92
Age, years, median (range)	33 (1-60)
Adults (age > 16 years)	80
Children (age ≤ 16 years)	12
Recipient sex	
Female	49
Male	43
Donor	
Sibling	45
HLA-matched (10/10) unrelated	35
MUD	12
Transplant material	
BM	14
PBPCs	78
Diagnosis	
Hematologic malignancies	78
Chronic myelogenous leukemia	11
Chronic lymphocytic leukemia	3
Acute myelogenous leukemia	25
Acute lymphoblastic leukemia	10
Other	29
Anemias and immunodeficiences	14
Conditioning regimen	
Myeloablative	36
RIC	56
aGVHD, grades	
0	43
I	14
II	15
III	10
IV	10
Chronic GVHD	
Extensive	25
Limited	11
CMV ≥100 DNA copies /10^5 cells	28
CMV IgG serostatus	
Recipient	
CMV IgG negative	8
CMV IgG positive	81
Donor	
CMV IgG negative	24
CMV IgG positive	64
HSCT	
Recipient IgG (+)/donor IgG(-)	21
Recipient IgG (-)/donor IgG(+)	5

MUD indicates matched unrelated donor; BM, bone marrow; PBPCs, peripheral blood progenitor cells; aGVHD, acute graft-versus-host disease; RIC, reduced intensity conditioning; HSCT, hematopoietic stem cell transplantation; CMV, cytomegalovirus.

The peak value of CMV copies aids in dividing patients into 2 subgroups: those with mild viremia usually not associated with apparent clinical complications and those with viremia at a level strongly associated with complications. Altogether, of the 92 patients in the study group, 46 were CMV-positive, and 28 of them had peak viremia exceeding 100 CMV DNA copies/10^5 cells. Clinical observations provided all pertinent information regarding immunosuppression and transplantation-related morbidity. Genetic data included microsatellite *IFNG* genotyping.

DNA Isolation and *IFNG* (CA)n Microsatellite Polymorphism Detection

DNA was extracted from PB using a QiAmp Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. *IFNG* microsatellite

polymorphism was analyzed as described previously [13]. In brief, a portion of the first intron of the *IFNG* gene, containing the (CA)n microsatellite region, was amplified using the TAMRA-labeled forward (5'-GCT GTC ATA ATA ATA TTC AGA C-3') and reverse (5'-CGA GCT TTA AAA GAT AGT TCC-3') primers. After denaturation at 95°C for 5 minutes, all of the samples were analyzed on 6% polyacrylamide gel with 7 M urea (Gibco, Paisley, UK) with an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Electrophoresis was carried out at 3000 V and 51°C. Genescan 3.1.2 software (Applied Biosystems) was used to analyze the electrophoretic patterns.

CMV DNA Quantification

The number of CMV DNA copies in PB cells was determined by real-time polymerase chain reaction (PCR) using the Opticon 2 system (MJ Research, Watertown, MA). The sequences of the PCR primers and the probe were selected from the *US17* region of CMV, and PCR was carried out as described by Machida et al. [14]. For a quantitative analysis of the number of CMV copies, a CMV standard was prepared by inserting the *US17* CMV gene fragment encoding viral DNA polymerase into a pCR 2·1 cloning vector (Invitrogen, Carlsbad, CA). A standard 5-point curve was obtained using serial dilutions (from 0 to 2.3×10^5 copies) of these CMV standards.

The initial number of blood cells and the number of β-actin copies were assessed for the quantification of the isolated DNA. This analysis was performed using a β-actin TaqMan Endogenous Control Kit (Eurogentec, Seraind, Belgium) according to the manufacturer's instructions. A 5-point β-actin standard curve was prepared using a serially diluted (from 0 to 1×10^4 copies) human Xsomal DNA control provided with the Eurogentec kit. The number of CMV genomic copies was calculated and related to the β-actin copies proportionally referring to 10^5 whole blood cells (WBCs).

Clinical Observation and Blood Phenotypic Analysis

Treatment-related toxicity and acute graft-versus-host disease (aGVHD) were diagnosed according to our standard operation procedure based on international GVHD diagnosis and staging criteria [12]. Residual disease and immunologic recovery were determined by flow cytometry. The panel of monoclonal antibodies (mAbs) for the detection of lymphocyte populations was purchased from Becton-Dickinson (San Jose, CA). A CD45 mAb was used to gate the lymphocyte populations. The cells were analyzed using a FACScalibur Flow Cytometer (Becton-Dickinson), using CellQuest software for data acquisition, and the data were analyzed using WinMDI 2.8 software. In most cases, 20,000 cells were acquired in the lymphocyte gate.

Retrospective analysis of the 92 patients revealed that those individuals with at least 100 copies of CMV DNA per 10^5 cells on one occasion suffered more frequently from post-HSCT complications, including sepsis, hepatitis, encephalitis, cystitis, pneumonia, hemolytic anemia or pure red cell aplasia, relapse or marrow failure, and/or aGVHD grade ≥ II, compared with those with absent or lower numbers of CMV DNA copies (0.46 vs 0.16; $P = .013$). This analysis does not prove that CMV is directly involved in the pathological mechanism of the aforementioned transplantation complications, but suggests an association between the presence of ≥ 100 CMV copies per 10^5 PB cells and post-HSCT morbidity. Therefore, 100 CMV DNA copies per 10^5 PB cells was chosen as the threshold indicating clinical significance. Indeed, the survival curves of patients having or lacking CMV reactivation at the level of ≥ 100 copies/10^5 cells differed significantly in favor of the latter group, with 41% and 71% ($P = .040$) of individuals surviving to 3 years post-HSCT, respectively.

Statistical Analysis

Statistical analysis was performed using CSS Statistica for Windows version 7.0 (Sta-Soft, Tulsa, OK). Univariate analyses of the *IFNG* genotype distribution in patients having or lacking CMV reactivation were performed using Fisher's exact test. The Mann-Whitney U test or Kruskal-Wallis nonparametric analysis of variance (ANOVA) were used to compute the differences between 2 or more independent patient groups, respectively. Factorial ANOVA was used to assess the possible association of 2 or more factors and logistic regression for multivariate analysis. Differences between samples were considered significant when $P < .05$.

RESULTS

CMV reactivation (≥ 100 CMV DNA copies/10^5 PB cells) was observed in 28 of the 92 HSCT recipients (30%) and was more frequent in those with < 10% $CD4^+$ lymphocytes compared with those with a higher percentage of $CD4^+$ lymphocytes in the blood (0.62 vs 0.21; $P = .001$). The patients with < 10% $CD4^+$ lymphocytes also had higher copy numbers than those with a higher percentage of $CD4^+$ lymphocytes (mean ± SEM, 4422 ± 1667 vs 937 ± 662 CMV copies/10^5 cells; $P < .001$). In addition, CMV reactivation with a higher number of copies was observed more often in patients (1) receiving a graft from not optimally matched donors (at a level below 10/10 HLA alleles) (0.67 vs 0.30; $P = .008$), (2) having aGVHD grade > I

Table 2. Univariate Analysis of Risk Factors for CMV Reactivation*

Factor		Cases with the Given Factor among Patients		*P* Value
		CMV Reactivation	Lack of CMV Reactivation	
Recipient *IFNG* genotype	Homozygous 13-CA-repeat genotype	11	11	.039
	Others	18	52	
aGVHD	Lack of aGVHD	12	31	.273
	aGVHD	20	29	
aGVHD grade ≥ II	Lack of aGVHD or aGVHD grade I	12	39	.075
	aGVHD grade ≥ II	17	24	
Conditioning regimen	Myeloablative	12	24	.820
	RIC	17	39	
Type of donor	MUD	18	30	.262
	Sibling	11	33	
Recipient age	<16 years	2	10	.327
	≥ 16 years	27	53	
Recipient CMV IgG	CMV IgG negative	2	6	.593
	CMV IgG positive	26	55	
Donor CMV IgG	CMV IgG negative	10	15	.453
	CMV IgG positive	19	44	
Donor-recipient IgG CMV serology mismatch	Mismatched (D+/R-, D-/R+)	12	14	.083
	Matched (D+/R+, D-/R-)	16	45	
	D-/R+	10	12	.185
	D+/R+, D-/R-, D+/R-	18	47	
	D-/R+	2	2	.598
	D+/R+, D+/R-, D-,R+	27	56	
Donor sex	Female	8	32	.044
	Male	21	31	
Recipient sex	Male	14	35	.653
	Female	15	28	
Source of HSCT	BM	5	9	.534
	PBPC	21	54	
$CD4^+$ lymphocytes	< 10%	13	9	.001
	≥ 10%	14	53	
HLA mismatch	Matched (10/10)	21	59	.008
	Mismatched	8	4	
Donor/recipient sex	Matched (female to female or male to male)	17	44	.345
	Mismatched (female to male or male to female)	12	19	
	Female to male	2	8	.496
	Male to male, female to female, and male to female	27	55	
	Male to female	9	11	.176
	Male to male, female to female, and female to male	20	52	

D+ indicates donor CMV IgG positive; D–, donor CMV IgG negative; R–, recipient CMV IgG negative; R+, recipient CMV IgG positive.
*CMV reactivation was defined as the presence of ≥ 100 CMV DNA copies/10^5 peripheral blood cells.

(0.41 vs 0.23; $P = .075$), and (3) having a different CMV IgG serostatus from that of the donor (0.46 vs 0.26; $P = .083$) (Table 2).

In total, 22 (24%) and 16 (17%) patients were homozygotes carrying 13 CA repeats (allele 3) and 12 CA repeats (allele 2) in the first intron of the *IFNG* gene, respectively. The rest of the patients were heterozygous for 12, 13, or more CA repeats. Compared with patients with other allele constellations, patients homozygous for the 13-CA-repeat *IFNG* genotype (*IFNG* 3/3 genotype) had higher peak values of CMV copies (4111 ± 1699 vs 950 ± 591 CMV copies/10^5 cells; $P = .041$) and experienced more CMV reactivation 0.5 vs 0.26; $P = .039$) (Figure 1A). These findings were seen in patients receiving myeloablative (MA) conditioning as well as in those receiving reduced-intensity conditioning (RIC); however, the difference was statistically significant only in the latter group (5873 ± 2374 vs 1092 ± 984 CMV copies/10^5 cells, $P = .001$; 0.67 vs 0.17, $P < .001$).

By univariate analysis, recipient sex, donor–recipient sex relation, type of donor (sibling or matched unrelated donor [MUD]), conditioning regimen (MA or RIC), CMV IgG donor and recipient serostatus, and recipient age had no significant influence on the risk of CMV reactivation (Table 2).

To evaluate the impact of genotypes composed of alleles with more than 13 repeats, the incidence of CMV reactivation in homozygotes carrying 13-CA repeats (allele 3) was compared with those carrying one 13-repeat allele as well as a second allele composed of more than 13 repeats. (Patients in both groups were homozygous for the presence of A at the polymorphic site.) The homozygotes for 13-CA repeats had CMV reactivation in 50% of cases; however, CMV reactivation was observed in only 14% of the cases in which the

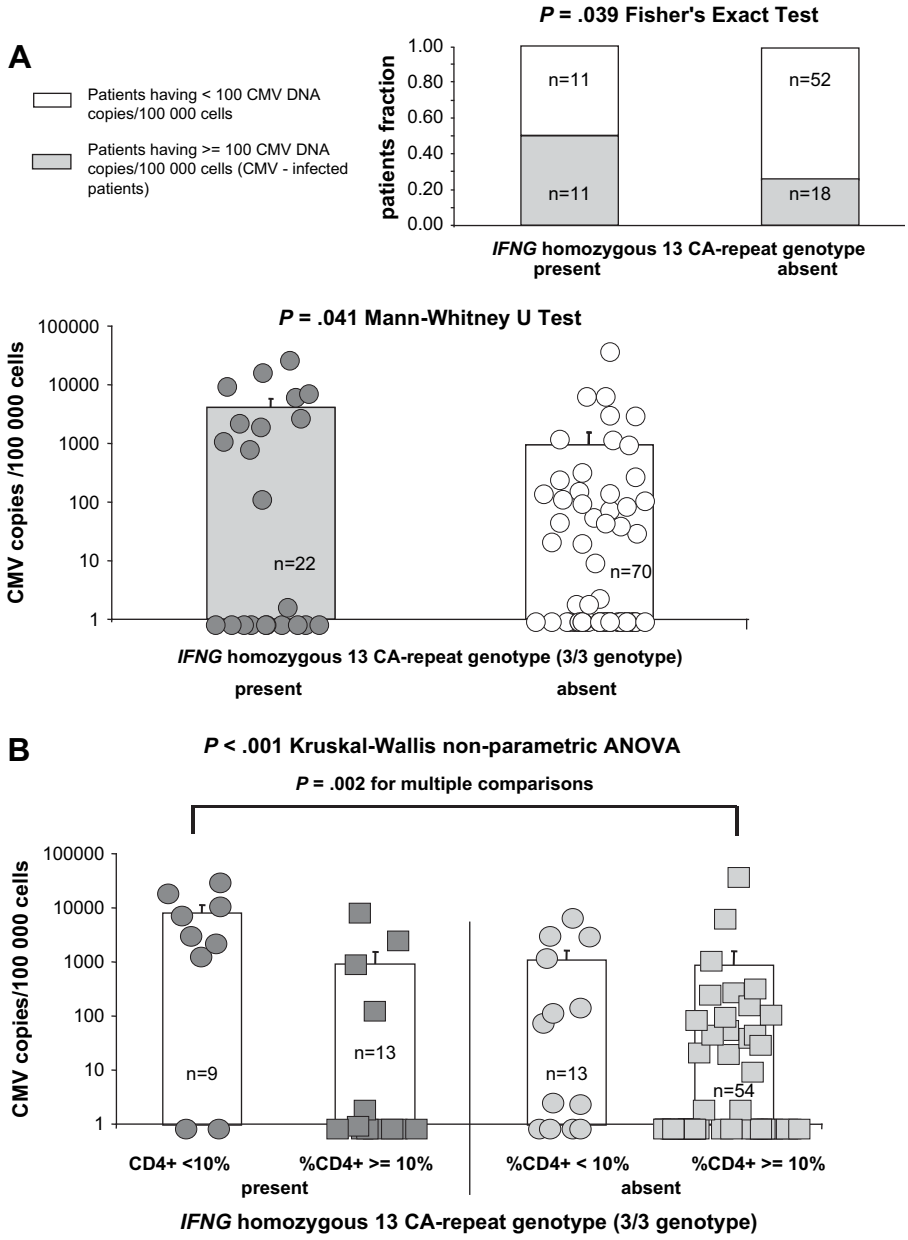

Figure 1. Relationship between the recipient *IFNG* polymorphism and the number of CMV copies and the incidence of CMV reactivation after HSCT (A) and the percentage of $CD4^+$ lymphocytes (B).

patient had one allele with 13 repeats and another allele with more than 13 repeats ($P = .18$), even though all of these patients were homozygotes for A at the *IFNG* +874 SNP site. Moreover, heterozygotes with respect to the CA-repeat polymorphism had less frequent reactivation of CMV compared with homozygotes for 13-CA-repeats (0.26 vs 0.50; $P = .059$).

Patients carrying the 13-CA-repeat homozygous *IFNG* genotype had more frequent viremia (≥ 100 copies/10^5 cells) for longer than 30 days compared with those with other allele constellations (0.72 vs 0.24; $P = .019$). In this group of homozygous patients, CMV viremia occurred early post-HSCT. Nine of the 22 13-CA-repeat *IFNG* genotype homozygotes, but only 12 of the 70 recipients with other alleles, were positive for CMV copies by 30 days post-HSCT ($P = .038$).

CMV seropositivity of the recipients was predictive of the risk of CMV viremia. To exclude the bias between HLA matching and CMV seropositivity in assessing the role of *IFNG* genotype as a risk factor of CMV reactivation, a subgroup of CMV-seropositive patients who also were fully HLA-matched was evaluated. In this group, the 13-CA-repeat homozygous *IFNG* genotype also was significantly associated with the presence of CMV copies in blood (0.53 vs 0.24; $P = .025$).

When examined 100 days post-HSCT, patients homozygous for the 13-CA-repeat genotype had a similar

proportion of $CD4^+$ lymphocytes as those with another type of allele constellation (13.5% ± 1.45% vs 18.1% ± 1.51% of $CD4^+$ lymphocytes; $P = .154$). Thus, the low-IFNG production genotype (ie, homozygotes for the 13-CA-repeat allele) did not influence the proportion of $CD4^+$ lymphocytes in the blood.

Compared with recipients with other *IFNG* genotypes and ≥ 10% $CD4^+$ lymphocytes, those with two 13-CA-repeat alleles (*IFNG* 3/3 genotype) and with < 10% $CD4^+$ lymphocytes had a significantly higher number of CMV copies (8547 ± 3628 vs 929 ± 756 CMV copies/10^5 cells; $P = .002$) (Figure 1B) and incidence of reactivation (0.78 vs 0.18; $P < .001$). Factorial ANOVA was used to assess whether homozygosity for the 13-CA-repeat genotype and a low proportion of $CD4^+$ lymphocytes might act in a synergistic fashion. The test showed that both factors were synergistic with respect to the number of CMV DNA copies ($P = .015$) during reactivation, but that the 2 factors appeared to act independently regarding the incidence of reactivation.

Patients homozygous for *IFNG* 13-CA repeats were more frequently positive for CMV copies regardless of whether 10 copies (0.50 vs 0.38; P = not significant [NS]) or 100 copies per 10^5 cells was considered. However, statistical significance was attained only at the 100-copy threshold value, (0.50 vs 0.26; $P = .039$). A low proportion of $CD4^+$ lymphocytes constituted a risk factor for CMV reactivation at levels of 10 copies/10^5 cells (0.38 vs 0.14; $P = .013$) as well as 100 copies/10^5 cells (0.62 vs 0.21; $P = .001$).

Gancyclovir given to decrease the risk of reactivation in patients receiving ATG did not affect the CMV reactivation rate with a high number of CMV DNA copies at later stages post-HSCT (0.31 vs 0.25; P = NS). Gancyclovir given as a preemptive treatment did not affect the frequency of CMV reactivation, which was usually noted before the treatment (0.38 vs 0.13; P = NS).

In univariate analysis, CMV positivity at the level of ≥ 100 DNA copies/10^5 cells was significantly associated with *IFNG* polymophism, aGVHD grade ≥ II, presence of negative IgG serology in the donor or recipient, and HLA mismatch (Table 2). All of these variables were included in multivariate analysis in addition to the following factors known to affect transplantation outcome: conditioning regimen, type of donor (matched sibling vs MUD), and female-to-male transplantation [15-18]. Multivariate logistic regression analysis showed that only homozygosity for the 13-CA-repeat genotype (odds ratio [OR] = 0.221; $P = .044$), $CD4^+$ lymphocyte level < 10% in the blood lymphocyte population (OR = 0.276; $P = .050$), and donor–recipient HLA mismatch (OR = 15.19; $P = .006$) played significant and independent roles in CMV reactivation with a high number of copies in patients post-HSCT (Table 3).

DISCUSSION

In the present study, CMV reactivation occurred in 30% of the patients, and the number of CMV DNA copies in these patients was in the range of 100 per 10^5 cells from 31 to 60 day post-HSCT. Similar data have been reported by Kim et al. [19], Ljungman et al. [20], Cortez et al. [21], Schulenburg et al. [22], and Busca et al. [23]. Our finding that patients with low percentages and numbers of $CD4^+$ lymphocytes were at increased risk for CMV reactivation also is in agreement with previous reports [24-26]. Consequently, our patient cohort behaved as expected with respect to the presence of and risk factors for CMV reactivation and thus was well suited for investigating unknown factors that might act in concert with known factors in contributing to CMV reactivation.

Viral copies were examined in the cellular pellet, which has a high clinical utility [27]. Comparative studies of plasma, whole blood, and leukocyte pellet have found a significantly lower level of CMV copies in plasma than in cell-containing samples [27-29]; thus, our study based on blood pellet nuclear cell examination better reflects the risk of CMV disease, demonstrating the actual number of copies residing in blood cells.

IFNG plays a crucial role in mounting an immune response against CMV [26,30,31]. The production of this cytokine depends on the number of cells able to respond to a given antigen and also is influenced by the polymorphic features of the *IFNG* gene that modulate the rate of transcription. In this study, we found that patients with CMV reactivation were more likely to have a genotype composed of alleles characterized by 13 CA repeats (*IFNG* 3/3 genotype) in the polymorphic region of the first intron of the *IFNG* gene. Indeed, it has been shown that the specific number of microsatellite repetitions can influence the rate of transcription [32-36].

The association between the number of CA repeats within the first intron of the *IFNG* gene and IFNG generation potential was originally described by Pravica et al. [7,8]. These authors reported that the allele with 12 CA repeats was associated with high IFNG generation potential. They also proposed a correlation between the 12-CA allele and a T nucleotide at position +874, in contrast to alleles having more than 12 repeats with A at this position. According to Pravica et al. [7,8], the presence of T, but not A, facilitates NFκB binding, thereby enhancing transcription.

Any interpretation of the association between gene polymorphic features and cytokine generation is usually complex, because the polymorphism can influence cytokine production directly or indirectly because of linkage disequilibrium with other known or unknown genetic factors. In an earlier study, we found that patients with sarcoidosis who had the tumor necrosis factor-alpha

Table 3. Multivariate Analysis of Risk Factors for CMV Reactivation

Factor		β coefficient	OR	95% CI	*P* value
Recipient IFNG genotype	Homozygous 13-CA-repeat genotype versus other	−1.509	0.221	0.0496-0.987	.044
aGVHD grade ≥ II	Lack of aGVHD or aGVHD grade I versus aGVHD grade ≥ II	0.560	1.751	0.488-6.279	.382
Conditioning regimen	RIC versus myeloablative	−0.066	0.936	0.275-3.180	.914
Type of donor	Sibling versus MUD	−1.036	0.355	0.075-1.682	.185
Donor–recipient serology mismatch	Mismatch versus match	0.737	2.090	0.518-8.438	.293
HLA mismatch	Mismatch versus match	2.721	15.195	2.103-109.818	.006
Recipient sex	Male versus female	−0.971	0.379	0.114-1.262	.108
Donor–recipient sex	Matched or male to female versus female to male	0.638	1.893	0.471-7.609	.361
$CD4^+$ lymphocytes	<10% versus ≥10%	−1.287	0.276	0.075-1.013	.049

Negative β coefficients for factors corresponding with *IFNG* genotype (β_1 = -1.509) and the percentage of $CD4^+$ cells (β_8 = -1.287) and positive β coefficients for HLA mismatch (β_6 = -2.721) show that *IFNG* genotypes other than the 13-CA-repeat homozygous genotype, as well as a high percentage (≥ 10%) of $CD4^+$ lymphocytes and HLA donor–recipient matching, decrease the risk of CMV reactivation. The ORs of these factors shows that the probabilities of CMV infection in patients lacking the *IFNG* 13-CA-repeat homozygous genotype, having ≥ 10% of $CD4^+$ cells, or being HLA matched (at the allele level) are decreased 4.5, 3.6, or 15.2 times, respectively.

(TNFA) 2 allele [37] were more susceptible to symptoms of Löfgren's syndrome. This allele correlates with higher TNFA production [38], but because of its strong linkage disequilibrium with HLA-A1 B8 DRB1*03 [39], it may be indirectly associated with low IFNG production [5,6]. The role of *IFNG* polymorphism in this disease was subsequently documented by findings demonstrating that the 13-CA-repeat *IFNG* genotype (3/3) is a risk factor for Löfgren's symptoms in patients with sarcoidosis [40]. This shows that the 13-CA-repeat homozygous genotype is associated with a disease in patients with sarcoidosis characterized by low IFNG production [41]. Interestingly, only the *IFNG* 3/3 genotype, not alleles with more than 12 CA repeats, is associated with symptoms of Löfgren's syndrome [40]. If Pravica et al. [7,8] are correct, then all patients with more than 12 CA repeats should behave in a manner similar to those having an A nucleotide at position +874, which, they postulate, is responsible for low IFNG generation potential. In the present study, the risk of CMV reactivation was associated with the *IFNG* 13-CA-repeat homozygous genotype, but not with other microsatellite genotypes having A at position +874. Thus, the presence of 13 repeats is associated with clinical situations known to be linked to low IFNG production. Similar observations came from our previous study, in which the 13-CA-repeat homozygous genotype (*IFNG* 3/3 genotype), but not 13/14, 13/15, or 14/14 CA repeats, was prevalent in patients with chronic GVHD (cGVHD) [42]. It is known that IFNG knockout mice develop aGVHD [43]; therefore, IFNG is not needed for GVHD manifestation, and a hypothetically low IFNG production genotype may facilitate cGVHD symptoms by making the host more susceptible to infections. Indeed, 13-CA-repeat homozygous patients undergoing HSCT were found to experience more frequent Epstein-Barr virus (EBV) reactivation than their counterparts with other constellations of CA microsatellite alleles [44]. Our observation regarding CMV reactivation agrees well with previous findings in individuals homozygous for the 13-CA-repeat allele and possibly lacking adequate IFNG production. Thus, these patients are susceptible to diseases in which low IFNG production facilitates CMV reactivation. Indeed, Miyake et al. [9] showed that cells homozygous for the 13-CA-repeat allele produce significantly lower amounts of IFNG compared with cells with other numbers of CA repeats. Findings reported by Abbott et al. [45] are in line with the hypothesized clinical relevance of the homozygous 13-CA-repeat genotype as a marker for low IFNG generation potential. These authors found that CA microsatellite polymorphism is associated with total serum IgE, and that a clinically relevant immune response to hepatitis B virus (HBV) is of greater significance than +874 T to A substitution. Thus, we postulate that microsatellite profile is important in shaping a successful immune response to CMV, similar to observations made with EBV and HBV [44,45].

Our present findings are especially noteworthy because the *IFNG* gene profile of the host represents a risk factor for CMV reactivation early post-HSCT. This also was observed with respect to the risk of aGVHD [13]. Interleukin (IL)-6, IL-10, transforming growth factor (TGF)-beta1, IL-2, and TNFA polymorphisms in the recipient also have been found to influence the risk of aGVHD [46-50]. Thus, host polymorphisms of some cytokine genes are important to HSCT outcome. Our interpretation of these findings is that the recipient *IFNG* genotype acts at the pretransplantation stage, making the host more susceptible to CMV subclinical reactivation associated with an increase in viral load. CMV IgG seropositivity in patients reflects the presence of primary infection, and further reactivations may result from one of the risk factors of post-HSCT reactivation [51,52].

Our finding of an association between the disparity in CMV serology in donor–recipient pairs and the risk of CMV infection may be interpreted as follows:

(1) A recipient, if positive, harbors CMV in a latent phase because of a primary infection, and the negative donor transplant material lacks memory cells specific to CMV [53]; and (2) a negative recipient lacks the immune system competence for CMV, which facilitates reactivation of the virus present in the transplant material [54]. Thus, reactivation is poorly controlled in these 2 situations. Indeed, patients receiving a transplant from a CMV-negative donor and CMV-negative patients receiving a transplant from a CMV-positive donor suffer more frequently from CMV reactivation (Table 2).

This putative high viral load in patients with the low–IFNG production genotype is associated with a higher incidence and number of CMV copies when the patients have a low $CD4^+$ lymphocyte fraction and number. Another point that merits discussion is the lack of association between donor IFNG genotype (individual data not shown) and the risk of CMV reactivation. We presume that the immune system impairment post-HSCT results from immunosuppression, and that the alloreactive hit is so severe that the weight of genetic factors influencing IFNG production are not strong enough to be valid in this situation.

The present study contributes to the literature of CMV reactivation in patients post-HSCT with the novel observation regarding the role of the homozygous 13-CA-repeat *IFNG* genotype of the host and the proportions of $CD4^+$ lymphocytes post-HSCT in CMV reactivation at a clinically significant level. Indeed, we found that patients with $\geq$ 100 CMV DNA copies suffered more frequently from treatment-related complications. A low proportion of $CD4^+$ lymphocytes is the sole factor in CMV reactivation, recognized as the presence of any number of CMV copies. *IFNG* genotype influences the pace of viral replication, which is significantly associated with a peak number of CMV DNA copies exceeding 100 per 10^5 peripheral blood cells. Thus, both factors act independently (see multivariate analysis), additively favoring the pace of replication.

The value of 100 copies per 10^5 cells was chosen to delineate a threshold value predictive of CMV reactivation–associated complications, including GVHD, relapses, and infections. The latter 2 complications likely are from the immunosuppressive potential of the CMV reactivation–associated process [55-57]. We found that a low proportion of $CD4^+$ lymphocytes favors early reactivation events, but *IFNG* genotype influences the incidence of reactivation with a high number of copies; thus, low IFNG producers more frequently have $\geq$ 100 CMV copy numbers per 10^5 blood cells. This level was found to be a threshold for clinically apparent complications that could be attributed directly or indirectly to CMV reactivation.

The hypothesis that *IFNG* genotype facilitates viral replication is also supported by the positive association between 13-repeat homozygosity and the presence of prolonged viremia. Determining the *IFNG* genotype before transplantation may help tailor viral surveillance measures post-HSCT, thereby preventing life-threatening complications associated with and facilitated by CMV.

ACKNOWLEDGMENTS

Financial disclosure: This work was supported by Grants N401 169 32/3358 and N N402 19 3335 from the Polish Ministry of Science and Higher Education.

REFERENCES

1. Bego MG, St Jeor S. Human cytomegalovirus infection of cells of hematopoietic origin: HCMV-induced immunosuppression, immune evasion, and latency. *Exp Hematol*. 2006;34:555-570.
2. Fraser GA, Walker II, Canadian Blood, and Marrow Transplant Group. Cytomegalovirus prophylaxis and treatment after hematopoietic stem cell transplantation in Canada: a description of current practices and comparison with Centers for Disease Control/Infectious Diseases Society of America/American Society for Blood and Marrow Transplantation guideline recommendations. *Biol Blood Marrow Transplant*. 2004;10:287-297.
3. Davis AM, Hagan KA, Matthews LA, et al. Blockade of virus infection by human $CD4^+$ T cells via a cytokine relay network. *J Immunol*. 2008;180:6923-6932.
4. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol*. 2007;96:41-101.
5. Petrovsky N, Harrison LC. HLA class II–associated polymorphism of interferon-γ production implications for HLA–disease association. *Hum Immunol*. 1997;53:12-16.
6. Lange A. Genetic factors predicting IFN-gamma generation potential in patients with sarcoidosis and after haematopoietic stem cell transplantation. *Int J Immunogenet*. 2008;35:385-388.
7. Pravica V, Perrey C, Stevens A, et al. A single nucleotide polymorphism in the first intron of the human IFN-gamma gene: absolute correlation with a polymorphic CA microsatellite marker of high IFN-gamma production. *Hum Immunol*. 2000; 61:863-866.
8. Pravica V, Asderakis A, Perrey C, et al. In vitro production of interferon-gamma correlates with CA repeat polymorphism in the human *IFN-GAMMA* gene. *Eur J Immunogenet*. 1999;26:1-3.
9. Miyake K, Nakashima H, Akahoshi M, et al. Genetically determined interferon-gamma production influences the histological phenotype of lupus nephritis. *Rheumatology (Oxford)*. 2002;41: 518-524.
10. Lee JY, Goldman D, Piliero LM, et al. Interferon-gamma polymorphisms in systemic lupus erythematosus. *Genes Immunol*. 2001;2:254-257.
11. Saha A, Dhir A, Ranjan A, et al. Functional IFNG polymorphism in intron 1 in association with an increased risk to promote sporadic breast cancer. *Immunogenetics*. 2005;57:165-171.
12. Apperley J, Carreras E, Gluckman E, et al. In: *Haematopoietic Stem Cell Transplantation—ESH/EBMT Handbook*, 5th ed. Genoa, Italy: Forum Service Editore; 2008 p. 592.
13. Mlynarczewska A, Wysoczanska B, Karabon L, et al. Lack of IFN-gamma 2/2 homozygous genotype independently of recipient age and intensity of conditioning regimen influences the risk of aGVHD manifestation after HLA-matched sibling haematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2004; 34:339-344.

14. Machida U, Kami M, Fukui T, et al. Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. *J Clin Microbiol*. 2000;38: 2536-2542.
15. Gandhi MK, Khanna R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect Dis*. 2004;4:725-738.
16. Mohty M, Jacot W, Faucher C, et al. Infectious complications following allogeneic HLA-identical sibling transplantation with antithymocyte globulin–based reduced intensity preparative regimen. *Leukemia*. 2003;17:2168-2177.
17. Chakrabarti S, Mackinnon S, Chopra R, et al. High incidence of cytomegalovirus infection after nonmyeloablative stem cell transplantation: potential role of Campath-lH in delaying immune reconstitution. *Blood*. 2002;99:4357-4363.
18. Bjorklund A, Aschan J, Labopin M, et al. Bone marrow risk factors for fatal infectious complications developing late after allogeneic stem cell transplantation. *Transplantation*. 2007;40: 1055-1062.
19. Kim DH, Kim JG, Lee NY, et al. Risk factors for late cytomegalovirus infection after allogeneic stem cell transplantation using HLA-matched sibling donor: donor lymphocyte infusion and previous history of early CMV infection. *Bone Marrow Transplant*. 2004;34:21-27.
20. Ljungman P, de La Camara R, Milpied N, et al. Randomized study of valacyclovir as prophylaxis against cytomegalovirus reactivation in recipients of allogeneic bone marrow transplants. *Blood*. 2002;99:3050-3056.
21. Cortez KJ, Fischer SH, Fahle GA, et al. Clinical trial of quantitative real-time polymerase chain reaction for detection of cytomegalovirus in peripheral blood of allogeneic hematopoietic stem-cell transplant recipients. *J Infect Dis*. 2003;188: 967-972.
22. Schulenburg A, Watkins-Riedel T, Greinix HAT, et al. CMV monitoring after peripheral blood stem cell and bone marrow transplantation by pp65 antigen and quantitative PCR. *Bone Marrow Transplant*. 2001;28:765-768.
23. Busca A, de Fabritiis P, Ghisetti V, et al. Oral valganciclovir as preemptive therapy for cytomegalovirus infection post–allogeneic stem cell transplantation. *Transpl Infect Dis*. 2007;9: 102-107.
24. Hakki M, Riddell SR, Storek J, et al. Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. *Blood*. 2003;102:3060-3067.
25. Almyroudis NG, Jakubowski A, Jaffe D, et al. Predictors for persistent cytomegalovirus reactivation after T-cell–depleted allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis*. 2007;9:286-294.
26. Harari A, Zimmerli SC, Pantaleo G. Cytomegalovirus (CMV)-specific cellular immune responses. *Hum Immunol*. 2004;65: 500-506.
27. Razonable RR, Brown RA, Wilson J, et al. The clinical use of various blood compartments for cytomegalovirus (CMV) DNA quantitation in transplant recipients with CMV disease. *Transplantation*. 2002;73:968-973.
28. Mengelle C, Sandres-Sauné K, Pasquier C, et al. Automated extraction and quantification of human cytomegalovirus DNA in whole blood by real-time PCR assay. *J Clin Microbiol*. 2003; 41:3840-3845.
29. Boivin G, Handfield J, Toma E, et al. Comparative evaluation of the cytomegalovirus DNA load in polymorphonuclear leukocytes and plasma of human immunodeficiency virus–infected subjects. *J Infect Dis*. 1998;178:1544-1546.
30. Ouyang Q, Wagner WM, Wikby A, et al. Compromised interferon gamma (IFN-gamma) production in the elderly to both acute and latent viral antigen stimulation: contribution to the immune risk phenotype? *Eur Cytokine Netw*. 2002;13: 392-394.
31. Avetisyan G, Larsson K, Aschan J, et al. Impact on the cytomegalovirus (CMV) viral load by CMV-specific T-cell immunity in recipients of allogeneic stem cell transplantation. *Bone Marrow Transplant*. 2006;38:687-692.
32. Brandt B, Meyer-Staeckling S, Schmidt H, et al. Mechanisms of *egfr* gene transcription modulation: relationship to cancer risk and therapy response. *Clin Cancer Res*. 2006;12:7252-7260.
33. Suzuki M, Kageyama S, Shinmura K, et al. Inverse relationship between the length of the EGFR CA repeat polymorphism in lung carcinoma and protein expression of EGFR in the carcinoma. *J Surg Oncol*. 2008;98:457-461.
34. Beilin J, Ball EM, Favaloro JM, et al. Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. *J Mol Endocrinol*. 2000;25:85-96.
35. Agarwal AK, Giacchetti G, Lavery G, et al. CA-repeat polymorphism in intron 1 of HSD11B2: effects on gene expression and salt sensitivity. *Hypertension*. 2000;36:187-194.
36. González AA, Carvajal CA, Riquelme E, et al. A polymorphic GT short tandem repeat affecting beta-ENaC mRNA expression is associated with low-renin essential hypertension. *Am J Hypertens*. 2007;20:800-806.
37. Seitzer U, Swider C, Stüber F, et al. Tumour necrosis factor alpha promoter gene polymorphism in sarcoidosis. *Cytokine*. 1997;9:787-790.
38. Wilson AG, Symons JA, McDowell TL, et al. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc. Natl. Acad. Sci. USA*. 1997; 94:3195-3199.
39. Wilson AG, de Vries N, Pociot F, et al. An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. *J Exp Med*. 1993;177:557-560.
40. Wysoczanska B, Bogunia-Kubik K, Suchnicki K, et al. Combined association between IFN-gamma 3,3 homozygosity and DRB1*03 in Löfgren's syndrome patients. *Immunol Lett*. 2004;91:127-131.
41. Swider C, Laba A, Moniewska A, et al. Presence of mRNA for interferon-gamma (IFN-gamma) in blood mononuclear cells is associated with an active stage I sarcoidosis. *Clin Exp Immunol*. 1995;100:401-405.
42. Bogunia-Kubik K, Mlynarczewska A, Wysoczanska B, et al. Recipient interferon-gamma 3/3 genotype contributes to the development of chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Haematologica*. 2005;90:425-426.
43. Puliaev R, Nguyen P, Finkelman FD, et al. Differential requirement for IFN-gamma in CTL maturation in acute murine graft-versus-host disease. *J Immunol*. 2004;173:910-919.
44. Bogunia-Kubik K, Mlynarczewska A, Jaskula E, et al. The presence of IFNG 3/3 genotype in the recipient associates with increased risk for Epstein-Barr virus reactivation after allogeneic haematopoietic stem cell transplantation. *Br J Haematol*. 2006; 132:326-332.
45. Abbott W, Gane E, Winship I, et al. Polymorphism in intron 1 of the interferon-gamma gene influences both serum immunoglobulin E levels and the risk for chronic hepatitis B virus infection in Polynesians. *Immunogenetics*. 2007;59:187-195.
46. Cavet J, Dickinson AM, Norden J, et al. Interferon-gamma and interleukin-6 gene polymorphisms associate with graft-versus-host disease in HLA-matched sibling bone marrow transplantation. *Blood*. 2001;98:1594-1600.
47. Karabon L, Wysoczanska B, Bogunia-Kubik K, et al. IL-6 and IL-10 promoter gene polymorphisms of patients and donors of allogeneic sibling hematopoietic stem cell transplants associate with the risk of acute graft-versus-host disease. *Hum Immunol*. 2005;66:700-710.
48. Noori-Daloii MR, Rashidi-Nezhad A, Izadi P, et al. Transforming growth factor-beta1 codon 10 polymorphism is associated with acute GVHD after allogenic BMT in Iranian population. *Ann Transplant*. 2007;12:5-10.
49. Bogunia-Kubik K, Wysoczanska B, Lange A. Non-HLA gene polymorphisms and the outcome of allogeneic hemato-poietic

stem cell transplantation. *Curr Stem Cell Res Ther*. 2006;1: 239-253.
50. Viel DO, Tsuneto LT, Sossai CR, et al. IL-2 and TNFA gene polymorphisms and the risk of graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. *Scand J Immunol*. 2007;66:703-710.
51. Craddock C, Szydlo RM, Dazzi F, et al. Cytomegalovirus seropositivity adversely influences outcome after T-depleted unrelated donor transplant in patients with chronic myeloid leukaemia: the case for tailored graft-versus-host disease prophylaxis. *Br J Haematol*. 2001;112:228-236.
52. Ljungman P, Brand R, Einsele H, et al. Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis. *Blood*. 2003;102:4255-4260.
53. Ljungman P. Risk assessment in haematopoietic stem cell transplantation: viral status. *Best Pract Res Clin Haematol*. 2007;20: 209-217.
54. Zhou W, Longmate J, Lacey SF, et al. Impact of donor CMV status on viral infection and reconstitution of multi-function CMV-specific T-cells in CMV-positive transplant recipients. *Blood*. 2009;113:6465-6476.
55. Fisher RA. Cytomegalovirus infection and disease in the new era of immunosuppression following solid organ transplantation. *Transpl Infect Dis*. 2009;11:195-202.
56. Miller-Kittrell M, Sparer TE. Feeling manipulated: cytomegalovirus immune manipulation. *Virol J*. 2009;6:4.
57. Mocarski ES Jr.. Immune escape and exploitation strategies of cytomegaloviruses: impact on and imitation of the major histocompatibility system. *Cell Microbiol*. 2004;6:707-717.